Low diversity of vegetative compatibility types and mating type of *Cryphonectria parasitica* in the southern Balkans

K. Sotirovski[a]*†, I. Papazova-Anakieva[a], N. J. Grünwald[b] and M. G. Milgroom[c]

[a]*Faculty of Forestry, University Ss. Kiril i Metodij, Skopje, Republic of Macedonia;* [b]*US Department of Agriculture-Agricultural Research Service, Prosser, WA 99350; and* [c]*Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA*

The diversity of vegetative compatibility (vc) types and mating type was estimated in populations of the chestnut blight fungus, *Cryphonectria parasitica*, throughout Macedonia and from selected areas in Greece. Nearly all of the 786 isolates (94%) from Macedonia were in a single vc type, EU-12; all 379 isolates from Greece were EU-12. Only six of 20 populations in Macedonia had more than one vc type. The diversity of vc types in the most diverse populations of Macedonia was comparable with the least diverse populations found previously in Italy. All but six of the 313 isolates assayed had the same mating type, *MAT-1*, and no perithecia of *Cryphonectria parasitica* were observed in any population. These results lead to the conclusion that sexual reproduction does not occur in these populations. The lack of vc type diversity may indicate a high potential for the spread of hypoviruses and successful biological control with transmissible hypovirulence. However, if sexual reproduction should occur in Macedonian populations, up to 32 vc types would be possible by recombination among vegetative incompatibility loci.

Keywords: *Cryphonectria parasitica*, *Cryphonectria radicalis*, genotypic diversity, mating type, vegetative incompatibility

Introduction

Some species of plant pathogens exhibit marked variation in diversity and population structure in different locations. In particular, some populations may be clonal, while others are sexual. For example, populations of *Phytophthora infestans* outside of Mexico until recently were clonal because only one mating type was present (Goodwin *et al*., 1994); and while most populations of *Magnaporthe grisea* are clonal, some populations in the Himalayas show evidence of recombination (Zeigler, 1998; Kumar *et al*., 1999). Among forest pathogens, population structure has been shown to change over time as epidemic fronts proceed, with older populations becoming more diverse, e.g. *Ophiostoma novo-ulmi* (Brasier, 1988) and *Neonectria coccinea* var. *faginata* (Mahoney *et al*., 1999).

Variation in the population structure of the chestnut blight fungus, *Cryphonectria parasitica*, is of particular interest because the diversity of vegetative compatibility (vc) types may be associated with the success of biological control by transmissible hypovirulence (Anagnostakis *et al*., 1986; Heiniger & Rigling, 1994; Milgroom, 1995; Cortesi *et al*., 2001). In general, the diversity of vc types of *Cryphonectria parasitica* is greater in North America than in Europe (Anagnostakis *et al*., 1986; Milgroom & Cortesi, 1999). Diversity is greatest in Asia, where *C. parasitica* is native, with almost every isolate having a unique vc type (Y.-C. Liu and M. G. Milgroom, unpublished data). The pattern of vc type diversity in *C. parasitica* in Europe shows that diversity is generally highest in areas where chestnut blight has been present for longer times, e.g. Italy, France, Switzerland and parts of Spain (Robin & Heiniger, 2001). In contrast, diversity is lowest where *C. parasitica* has been present for relatively short periods of time (Robin & Heiniger, 2001), e.g. Portugal (Gouveia *et al*., 2001), Switzerland north of the Alps (Hoegger *et al*., 2000) and Germany (as reported in Robin & Heiniger, 2001). In addition to duration of residence, the diversity of vc types in Italy was shown to be related to the prevalence of sexual structures (perithecia) of *C. parasitica* (Milgroom & Cortesi, 1999). As an extreme example, the population in Italy with the lowest vc type diversity, Zafferana (Cortesi *et al*., 1996), had no perithecia and only one mating type (Milgroom & Cortesi, 1999) so that sexual reproduction is not possible in this population (Marra & Milgroom, 2001). The lack of sexual reproduction relates to vc type diversity because recombination among vegetative incompatibility (*vic*) genes (Cortesi & Milgroom, 1998) increases or maintains the diversity of the multilocus genotypes that define vc types.

In the southern Balkan region of Europe, *C. parasitica* was first observed in Greece in 1964 (Xenopoulos, 1982)

*To whom correspondence should be addressed.

†E-mail: kirils@mt.net.mk

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and in the Republic of Macedonia in 1974 (V. Papazov, University Ss. Kiril i Metodij, Skopje, personal communication). Despite this length of time compared with more recent introductions in other parts of Europe, sampling of *C. parasitica* in one region of Greece (Mt Pelion) revealed only a single vc type (Xenopoulos, 1982; Cortesi *et al*., 1997), and only four were found in a more extensive survey (Perlerou, 2002). Interestingly, no perithecia and only one mating type were found in Greece (Xenopoulos, 1982), consistent with the low diversity of populations also observed in southern Italy (see above). Comparable studies of *C. parasitica* have not been reported previously, except in preliminary form (Sotirovski, 2000). The present study is aimed at quantifying vc type diversity in Macedonia and Greece.

A major constraint to comparing genotypic diversity among populations from different studies is that sampling is often conducted with different methods. The two most critical factors for comparing diversity estimates are the geographical scale at which samples are collected, and sample size. For example, some studies estimate diversity for local populations (single orchards or small plots), while others report diversity for entire regions or countries (Robin & Heiniger, 2001). Variation in sample size also complicates comparisons of diversity because sample size directly affects diversity estimates (Grünwald *et al*., 2003). Further complications in making comparisons of published data of diversity in *C. parasitica* arise because some studies report only the presence and absence of vc types but not their frequencies in each sample. For this reason, comparisons of vc type diversity in *C. parasitica* in this report are limited to studies using similar sampling strategies, with approximately equal sample sizes.

The overall objectives of this study were to determine the vc type and mating type diversity of *C. parasitica* in Macedonia and Greece. Specifically, our objectives were: (i) to estimate vc type diversity throughout Macedonia and in selected regions of Greece, and to compare these diversity estimates with other populations of *C. parasitica*; (ii) to estimate mating type frequencies in field populations of *C. parasitica*; and (iii) to determine whether perithecia of *C. parasitica* are present in Macedonia and Greece.

Materials and methods

Sampling and isolation

Cankers caused by *C. parasitica* on the European chestnut (*Castanea sativa*) were sampled from 20 populations, in eight regions throughout the Republic of Macedonia beginning in 1995, and from 10 populations in three regions of Greece in 2000 (Fig. 1). As in other studies (Cortesi *et al*., 1996; Milgroom & Cortesi, 1999), a population was defined in terms of the local site sampled; all sites were approximately 1–3 ha in size and at least 2 km from any other site. While this definition of a population is arbitrary, the local diversity of vc types is considered most relevant for biological control (Milgroom, 1995). Within each population, a piece of bark (~3×3 cm) was removed from one canker per tree to avoid sampling clones (Milgroom *et al*., 1991; Milgroom & Lipari, 1995). Bark samples were stored at –20°C until *C. parasitica* was isolated as described previously (Cortesi *et al*., 1996). Isolates were stored on glass fibre filters and stored at –20°C until used. From these samples, more than 1100 isolates in total were obtained. To determine whether *C. radicalis* was present in samples of *Cryphonectria* from chestnuts, as postulated by Cortesi *et al*. (1997), all isolates were grown on potato dextrose agar (PDA, Difco, Detroit, MI, USA) for 10–14 days in the laboratory (approx 21–22°C) under fluorescent lights (12 h daily) to observe their cultural characteristics. Under these conditions, *C. radicalis* produces pinkish mycelium, while *C. parasitica* does not. Isolates showing any trace of pink coloration were further assayed for restriction fragment length polymorphisms (RFLP) in the ITS region of nuclear ribosomal RNA as described previously (Myburg *et al*., 1999) to confirm their identity.

After isolation, each bark sample was examined under a dissecting microscope for the presence of perithecia. This often required dissection of several stromata per bark sample.

Vegetative compatibility assays

Isolates of *C. parasitica* were assayed for vegetative compatibility as described in Cortesi *et al*. (1996). Briefly, this assay was done by growing pairs of isolates at 25°C on PDA amended with bromocresol green (50 mg L^{-1}, Sigma, St Louis, MO, USA) (Powell, 1995). After 5–7 days, cultures were examined for the presence of a barrage, i.e. a zone of cell death between the colonies. Based on a survey of two Greek populations of *C. parasitica* in which vc type EU-12 was the dominant type found (Cortesi *et al*., 1997), all of the isolates were first compared with a tester isolate of EU-12 (Cortesi & Milgroom, 1998; Cortesi *et al*., 1998). Isolates incompatible with EU-12 were then tested against additional testers of other known vc types.

Mating type assays

In all, 179 and 134 isolates were assayed from Macedonia and Greece, respectively, for mating type using a PCR assay with primers M1-GS1 and M1-GS2-rev for *MAT-1* and primers M2-GS2 and InvA5n for *MAT-2* (Marra & Milgroom, 1999; McGuire *et al*., 2001). Culturing, DNA preparation and PCR assays were carried out exactly as has been described previously (Marra & Milgroom, 1999; McGuire *et al*., 2001).

Analysis of genotypic diversity

The diversity of vc types was estimated in each sample by comparing richness, evenness and total diversity (Grünwald *et al*., 2003). The richness component of diversity in this study was the observed number of vc types per sample. In this study, the maximum number of genotypes,

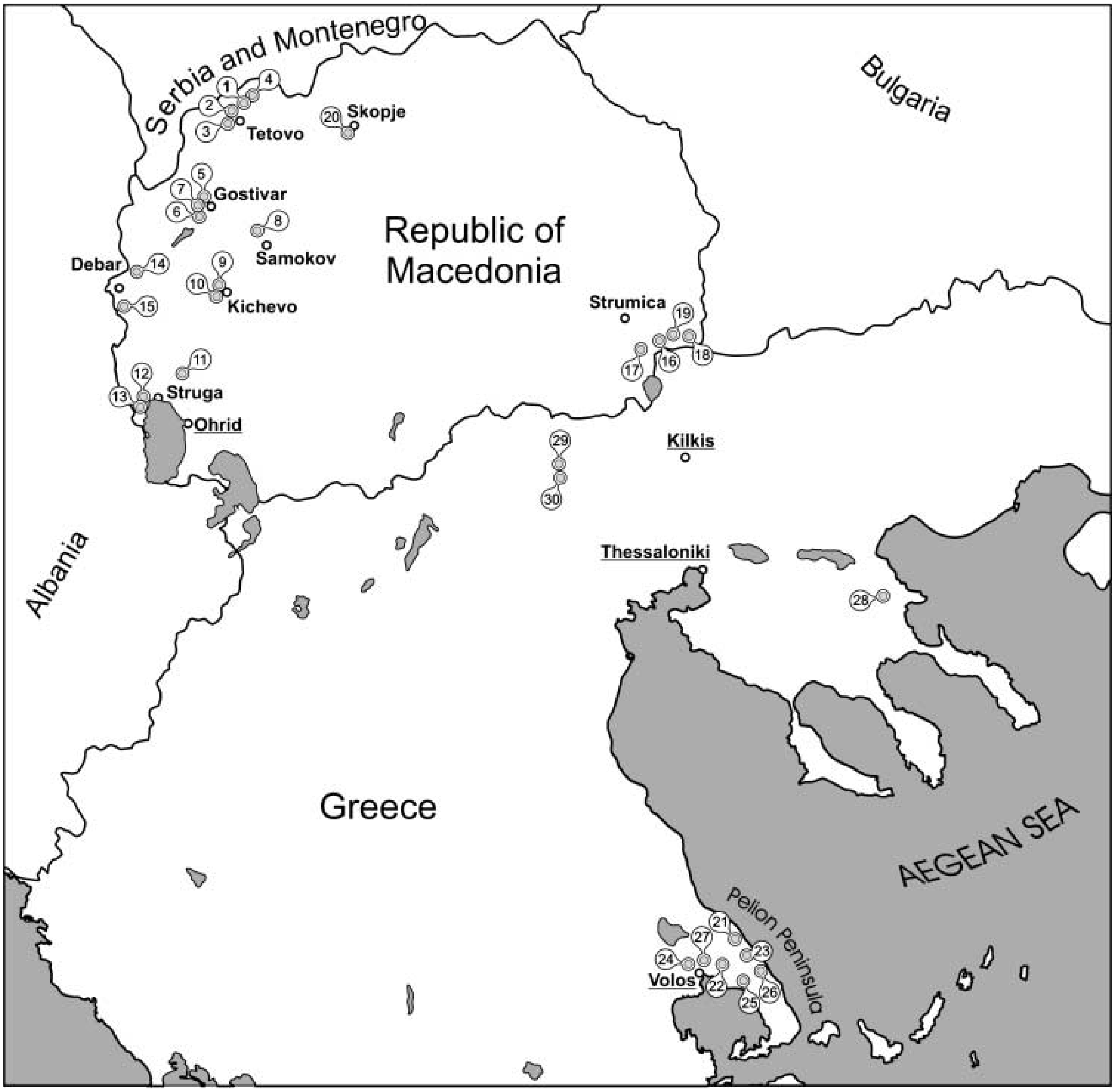

Figure 1 Sampling locations for *Cryphonectria parasitica* in Macedonia and Greece; underlined city names are for additional geographical reference. Numbers within circles in each region indicate populations sampled, as defined in Tables 1 and 2 for Macedonia and Greece, respectively.

g_{max}, was the potential number of multilocus *vic* genotypes (i.e. vc types), 2^k, where k is the number of polymorphic *vic* loci in the sample (Cortesi & Milgroom, 1998). To estimate genotypic diversity, indices H' and $\hat{G}$ were used as recommended by Grünwald *et al.* (2003), where H' is Shannon–Wiener's index, $H' = -\sum_i [p_i \ln(p_i)]$, p_i is the frequency of the ith vc type (Shannon & Weaver, 1949), and $\hat{G}$ is the genotypic diversity index presented in Stoddart & Taylor (1988), $\hat{G} = 1/\sum p_i^2$, which can also be calculated as $\hat{G} = 1/(\sum[(f_x)(x/n)^2]$, where n is the sample size, and f_x is the number of genotypes observed x times. The two indices differ in that $\hat{G}$ weights the number of abundant genotypes more strongly, whereas H' weights rarer genotypes more strongly (Grünwald *et al.*, 2003). To estimate evenness, the index E_5 was used as described in Grünwald *et al.* (2003), where $E_5 = (\hat{G} - 1)/(e^{H'} - 1)$.

To compare estimates of vc type diversity in Macedonia and Greece with studies done in other parts of Europe and in the USA, data from 11 populations in Italy (Cortesi *et al.*, 1996), two in Switzerland (Bissegger *et al.*, 1997) and three in the USA (Milgroom & Cortesi, 1999) were used. These populations were chosen for comparison because each one was roughly equivalent in terms of sampling methods as in the present study. To compare estimates of diversity between samples with different sizes, rarefaction analysis and bootstrapping were used as described previously (Grünwald *et al.*, 2003), as well as randomization tests. Rarefaction analysis was used to compare richness and was implemented using the program <Rarefac.c>. Bootstrapping was conducted using the SAS macro <jackboot.sas> (available on-line from the SAS Institute, Cary, NC, USA) modified to calculate indices of diversity and evenness. Bootstrapping was conducted using 2000 resamples at a confidence interval of 90% using the normal 'standard' confidence interval option in the SAS macro. Both rarefaction and bootstrapping were done to compare parameter estimates while holding sample sizes constant and equal to the smallest sample in Macedonia (see below). A randomization test (Solow, 1993; Manly, 1997) based on 10 000 samples was performed to assess whether H' was significantly different among populations. *P*-values were Bonferroni corrected for multiple comparisons.

Results

Diversity of vc types

The diversity of vc types was extremely low in both Macedonia and Greece. More than 780 isolates from Macedonia and 379 isolates from Greece were tested for vegetative compatibility and 96% of these (1116/1165) were in a single vc type, EU-12. Thirteen of the 20 populations in Macedonia and all 10 populations in Greece comprised isolates only in EU-12 (Tables 1 and 2). Four other vc types were found in Macedonia but were typically represented by a few isolates in each polymorphic population (Table 1). One population (Glogi) in the Tetovo region had four vc types, while a population (Osoj) in Kichevo had 49 isolates (68%) of EU-12 and 22 isolates (29%) of EU-2.

No vc type diversity was observed in most Macedonian and Greek populations; only six populations in Macedonia had more than one vc type. For four of the six populations in Macedonia with more than one vc type (all but Glogi and Osoj), diversity was significantly lower than in populations in northern Italy, Switzerland south of the Alps and the USA (Fig. 2), as reflected by the fact that 90% confidence intervals for H' and $\hat{G}$ do not overlap confidence intervals of these other populations (Table 3), and exact tests show significant differences for H' (Table 4). Diversity estimates in Glogi and Osoj were significantly lower than those in Switzerland and the USA, but they were significantly lower for only some of the populations

Table 1 Vegetative compatibility types and mating types of *Cryphonectria parasitica* samples collected in the Republic of Macedonia (1995–2000)

Region/ population	Year	*n*	vc type					Mating type	
			EU-12	EU-1	EU-2	EU-10	EU-22	*MAT-1*	*MAT-2*
Tetovo									
1[a] Glogi	1996	46	34	8 (4)[b]		1	3	25	4
2 Poroj	2000	63	59		1		3	1	0
3 Kale	1998, 2000	49	49					1	0
4 Vratnica	2000	49	46 (1)				3	1	1
Gostivar									
5 Galate	1996, 2000	57	52		4		1	21	0
6 Rechane	1997	8	8						
7 Vrutok	2000	44	43		1				
Samokov									
8 Lupshte	1996	43	43					17	0
Kichevo									
9 Osoj	1998	72	49	1	22			13	0
10 Ivani Dol	1998	10	10						
Struga									
11 Trebenishta	1997	56	56					17	0
12 Frangovo	1995, 1996	54	54 (1)					14	1
13 Kalishte	1995	9	9						
Debar									
14 Skudrinje	1996, 1997	53	53					20	0
15 Jepishte	1996	49	49					19	0
Strumica									
16 Drazhevo	1998	51	51					16	0
17 Bansko	1998	7	7					2	0
18 Smolare	1998	55	55					11	0
19 Mokrievo	1998	9	9						
Skopje									
20 Vodno	2000	2	1				1	–[c]	–
Total		786	737	9	28	1	11	173	6

[a]Population number used in Fig. 1 to show approximate location in Macedonia.
[b]Numbers in parentheses are the number of *MAT-2* isolates in this vc type.
[c]Not tested.

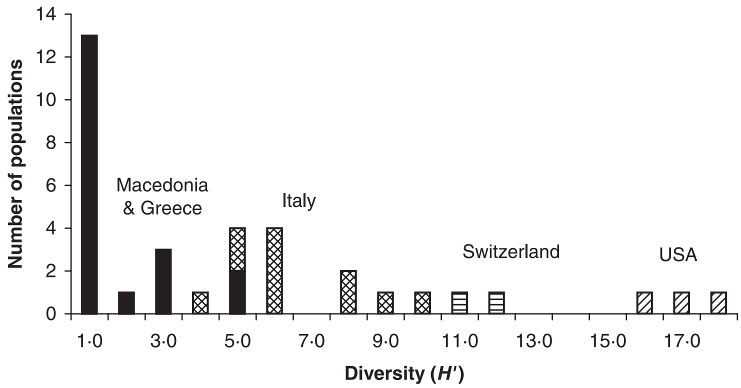

Figure 2 Distribution of vc type diversity estimates (H') in populations of *Cryphonectria parasitica* from Macedonia and Greece (solid bars; this study), and comparable studies in Italy (cross-hatched bars), Switzerland (horizontal stripes) and the USA (hatched bars) (Milgroom & Cortesi, 1999).

Table 2 Vegetative compatibility and mating types of *Cryphonectria parasitica* samples collected in Greece in 2000, and number of *C. radicalis* sampled in each population

Region/ population	vc type EU-12	Mating type		
		N[b]	*MAT*-1	*C. radicalis*
Mt. Pelion				
21[a] Anilio	44	41	41	0
22 Hania	61	–[c]	–	0
23 Kissos	83	–	–	1
24 Makrinitsa	15	15	15	16
25 Miles	28	27	27	0
26 Mouresi	36	34	34	0
27 Portaria	20	17	17	13
North-east				
28 Stageira	42	–	–	0
North				
29 Kastaneri	21	–	–	3
30 Griva	29	–	–	4
Total	379	134	134	37

[a]Population number used in Fig. 1 to show approximate location in Greece.
[b]Number of isolates assayed for mating type.
[c]Not tested.

in northern Italy (Tables 3 and 4). In contrast, populations in Macedonia with more than one vc type were generally comparable in richness and diversity to the least diverse populations in southern Italy (Tonara, Teano, Cittanova and Zafferana; Tables 3 and 4, Fig. 2). The polymorphic Macedonian populations had some of the lowest and the highest estimates of evenness (E_5), although their bootstrap confidence intervals were relatively large and overlapped those estimated from most Italian populations. The highest estimate of evenness was for Osoj where the two most common vc types occurred at frequencies of 68 and 29% (Table 1). Overall, however, populations of *C. parasitica* in Macedonia and Greece were less diverse than populations in Italy (Fig. 2).

Mating types and perithecia

Among the 179 isolates from Macedonia and 134 from Greece assayed for mating type, all but six Macedonian isolates were *MAT-1* (Table 1); all isolates assayed from Greece were *MAT-1* (Table 2). Five of the six *MAT-2* isolates were found in the Tetovo region, four of them in one population (Glogi). The sixth *MAT-2* isolate was found in Frangovo, in the Struga region (Fig. 1). The four *MAT-2* isolates from Glogi were in vc type EU-1; the other two (from Vratnica and Frangovo) were in EU-12 (Table 1).

No perithecia were observed in any of the bark samples from any population.

Occurrence of *Cryphonectria radicalis*

In Macedonia, only one isolate of *C. radicalis* was found on Belasica Mountain in the Strumica region. In Greece, a total of 37 isolates of *C. radicalis* were found in two regions (Table 2). In the Mt Pelion region, *C. radicalis* was found in three of the seven populations sampled. It was as common as *C. parasitica* in Makrinitsa, and nearly as common in Portaria, ~2 km away. Only one isolate of *C. radicalis* was found in Kissos, and none was found in other nearby populations in Mt Pelion. The remaining two populations (Kastaneri and Griva) where *C. radicalis* was found were in the north, close to the border with Macedonia (Fig. 1).

Discussion

The diversity of *C. parasitica* in Macedonia and Greece is among the lowest reported in this species. Nearly all of the isolates were in vc type EU-12, and 98% of all isolates assayed for mating type were *MAT-1*. In contrast, comparable samples of *C. parasitica* in the USA typically comprise 25 or more vc types (Milgroom *et al*., 1991; Milgroom & Cortesi, 1999), and in Japan and China, almost every isolate is in a unique vc type within local populations (Y.-C. Liu and M. G. Milgroom, unpublished data). The most diverse populations in Macedonia are comparable with the least diverse populations in Italy, all in the south (Cortesi *et al*., 1996), but markedly less diverse than populations in northern Italy or southern Switzerland (Table 3). The results reported here are similar to those found previously in the Mt Pelion region, in which only a single vc type was found (Xenopoulos, 1982; Cortesi *et al*., 1997); however, a total of four vc types have

Table 3 Richness, diversity and evenness of vc types of *Cryphonectria parasitica* in Macedonia, Italy, Switzerland and USA

		Richness[b]			Diversity[c]				Evenness[c]	
					H'		$\hat{G}$			
Population[a]	N	g_{obs}	E (g_{44})	g_{max}	H'	90% CI	$\hat{G}$	90% CI	E_5	90% CI
Macedonia										
Glogi	46	4	3·98	32	0·789	(0·558, 1·020)	1·720	(1·325, 2·116)	0·600	(0·481, 0·719)
Poroj	63	3	2·66	8	0·272	(0·071, 0·474)	1·137	(0·994, 1·280)	0·438	(0·000, 1·046)
Vratnica	49	2	2.00	4	0·230	(0·062, 0·399)	1·130	(0·994, 1·266)	0·502	(0·000, 1·137)
Galate	57	3	2·76	8	0·341	(0·131, 0·552)	1·194	(1·018, 1·370)	0·477	(0·075, 0·880)
Vrutok	44	2	1·96	8	0·109	(0·000, 0·247)	1·047	(0·969, 1·124)	0·406	(0·000, 1·652)
Osoj	72	3	2·61	16	0·684	(0·543, 0·824)	1·796	(1·518, 2·075)	0·812	(0·670, 0·953)
Italy										
Donnaz	50	4	3·89	8	0·961	(0·780, 1·142)	2·244	(1·837, 2·651)	0·771	(0·663, 0·879)
Crevoladossola	131	10	6.81	16	1·542	(1·317, 1·767)	3·658	(2·873, 4·443)	0·723	(0·617, 0·830)
Valtellina	46	8	7·93	32	1·374	(1·106, 1·641)	2·792	(2·028, 3·556)	0·607	(0·495, 0·720)
Bergamo	158	16	9·11	32	1·761	(1·477, 2·045)	3·958	(2·957, 4·958)	0·614	(0·526, 0·702)
Pigna	48	6	5·78	16	0·982	(0·709, 1·254)	1·889	(1·386, 2·391)	0·532	(0·421, 0·644)
Corniglio	50	4	3·98	32	0·816	(0·589, 1·043)	1·781	(1·379, 2·182)	0·619	(0·492, 0·746)
Pomina	50	7	6·75	32	1·358	(1·124, 1·592)	2·990	(2·309, 3·672)	0·689	(0·594, 0·785)
Tonara	33	5	3·97[d]	32	0·874	(0·596, 1·152)	1·694	(1·261, 2·127)	0·497	(0·408, 0·585)
Teano	194	7	3·31	64	0·659	(0·435, 0·884)	1·542	(1·239, 1·845)	0·580	(0·430, 0·731)
Cittanova	50	4	3·77	16	0·712	(0·506, 0·919)	1·676	(1·341, 2·010)	0·650	(0·506, 0·795)
Zafferana	50	2	2·00	8	0·405	(0·244, 0·566)	1·317	(1·102, 1·533)	0·635	(0·489, 0·781)
Switzerland										
Lumino	86	14	10·48	32	1·944	(1·684, 2·204)	4·821	(3·488, 6·155)	0·638	(0·534, 0·743)
Gnosca	62	16	13·35	32	2·181	(1·911, 2·451)	5·951	(4·178, 7·723)	0·630	(0·520, 0·741)
USA										
Finzel	57	26	22·21	128	2·940	(2·709, 3·171)	13·944	(10·646, 17·242)	0·723	(0·624, 0·821)
Bartow	59	30	25·03	128	3·139	(2·918, 3·359)	17·318	(13·506, 21·131)	0·739	(0·638, 0·841)
Depot Hill	58	34	28·41	128	3·317	(3·108, 3·526)	21·097	(16·796, 25·398)	0·756	(0·649, 0·864)

[a]Populations from Macedonia represent only those with more than one vc type (Table 1). Data for Italy, Switzerland and the USA are from Cortesi *et al*. (1996), Bissegger *et al*. (1997), and Milgroom & Cortesi (1999), respectively.
[b]Richness is expressed in terms of the number of observed vc types (g_{obs}), number expected ($E\,[g_{44}]$) by rarefaction analysis (Grünwald *et al*., 2003) for the smallest sample in Macedonia ($n = 44$), and maximum number (g_{max}) possible assuming recombination of all *vic* loci.
[c]Diversity and evenness were estimated as described in Grünwald *et al*. (2003). Confidence intervals were estimated by bootstrapping for sample sizes of $n = 44$.
[d]Sample size for Tonara is smaller than the smallest sample size for Macedonia, therefore richness was estimated for $n = 33$.

been reported throughout Greece (Robin & Heiniger, 2001; Perlerou, 2002). No previous reports of vc types were available for Macedonia, although other reports from eastern Europe and Turkey are variable. For example, typically one to three vc types per population were found in Hungary (Radócz, 2001) and Slovakia (Juhásová & Bernadovicová, 2001). In Bosnia-Herzegovina, only one to three vc types were found in the eastern, central and south-western regions, but 29 were found in the north-western region (Trestic *et al*., 2001). In Turkey, only one vc type (EU-1) has been found (Gurer *et al*., 2001), except in one population that has a few isolates of EU-12 (Çeliker & Onogur, 2001). However, comparisons of vc type diversities with these other studies are difficult to interpret because of differences in the geographical scale of sampling and numbers of isolates collected per sample (Robin & Heiniger, 2001), both of which markedly affect diversity estimates (Grünwald *et al*., 2003). However, by comparing samples in Macedonia and Greece with studies done with comparable sampling methods (Cortesi *et al*., 1996; Bissegger *et al*., 1997; Milgroom & Cortesi, 1999), it was possible to compare diversity among different regions.

Within the six Macedonian populations with more than one vc type, the number of vc types sampled (richness, g_{obs}) is less than expected based on the number of polymorphic *vic* loci. Because the underlying genetics of vegetative incompatibility are known for nearly all vc types in Europe (Cortesi & Milgroom, 1998; Robin *et al*., 2000), it is possible to determine the potential number of multilocus *vic* genotypes (vc types) for each population (g_{max}, Table 3). The findings that g_{obs} is consistently less than g_{max}, the lack of polymorphism for mating type, and the absence of perithecia in Macedonia and Greece indicate that *C. parasitica* is not reproducing sexually in these populations. These results, together with the fact that EU-12 is the dominant vc type in populations in southern Italy and eastern Europe (Robin & Heiniger, 2001), raise the question as to whether these *C. parasitica* populations are

Table 4 *P*-values for *t*-test[a] comparing estimates of diversity (H') among polymorphic Macedonian populations of *Cryphonectria parasitica* (i.e. with more than one vc type, $H' > 0$), and between these populations and those sampled in Italy, Switzerland, and the USA (see Table 3)

	Polymorphic Macedonian populations					
Population	Glogi	Poroj	Vratnica	Galate	Vrutok	Osoj
Macedonia						
Glogi	–					
Poroj	0·0135	–				
Vratnica	0·0027	0·7021	–			
Galate	0·0317	0·6937	0·395	–		
Vrutok	0·0005	0·3056	0·3463	0·0797	–	
Osoj	0·5412	0·0009	0·0022	0·0149	< 0·0001	–
Italy						
Donnaz	0·2384	< 0·0001	< 0·0001	< 0·0001	< 0·0001	0·0046
Crevoladossola	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
Valtellina	0·0059	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
Bergamo	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
Pigna	0·3110	< 0·0001	< 0·0001	< 0·0001	< 0·0001	0·0102
Corniglio	0·8337	< 0·0001	< 0·0001	0·0001	< 0·0001	0·1412
Pomina	0·0055	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
Tonara	0·6599	0·0015	< 0·0001	0·0033	< 0·0001	0·2189
Teano	0·4243	0·002	0·0035	0·0238	0·0004	0·8376
Cittanova	0·6783	0·0153	0·0138	0·0454	0·0028	0·8637
Zafferana	< 0·0001	0·2168	< 0·0001	0·7487	< 0·0001	0·0003
Switzerland						
Lumino	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
Gnosca	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
USA						
Finzel	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
Bartow	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001
Depot Hill	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001	< 0·0001

[a]*P*-values are based on two-sided *t*-tests from 10 000 randomizations (Solow, 1993; Manly, 1997). Because we made a total of 111 pairwise comparisons between populations, we used a Bonferroni correction such that a *P*-value less than 0·005 is considered significantly different at the $\alpha = 0{\cdot}05$ level of significance.

clonal, with a single clone having colonized and dominated in these areas. Testing this hypothesis, however, will require the use of a different set of genetic markers besides vc type. Previous studies have shown that polymorphisms for DNA fingerprinting are common within vc types and therefore vc types do not necessarily represent clones (Liu *et al*., 1996). If EU-12 does represent a clone of *C. parasitica*, it may be the dominant type in southern and eastern Europe because of chance (i.e. a founder effect), or because this clone is adapted to conditions in these areas. It appears that EU-12 may have been the dominant vc type in Greece for at least 20 years. Xenopoulos (1982) reported finding only one vc type in Mt Pelion, which was identified as vc56 and later shown to be the same as EU-12 (Cortesi *et al*., 1997). Furthermore, all isolates from Greece were mating type a (Xenopoulos, 1982), which has been renamed *MAT-1* based on homology to mating type genes in other ascomycetes (Marra & Milgroom, 2001). *C. parasitica* was first found in Greece in 1964 and in Macedonia in 1974. It is speculated that the same clone has been present since its introduction, and if so, the population structure of the epidemic front in the southern Balkans appears to have been stable for many years. The high diversity of vc types found in north-western Bosnia-Herzegovina (Trestic *et al*., 2001) raises the question as to whether diversity in the southern Balkans will increase in the future as *C. parasitica* migrates further south in a second wave of migration, as may be occurring already in the area near Glogi. Alternatively, if sexual reproduction occurs, e.g. in a population like Glogi where both mating types occur, then recombination could result in much greater diversity even in the absence of further immigration. Intensive monitoring of vc type and mating type polymorphisms are needed to follow the fate of these populations.

A previous study in Greece reported the presence of another species of *Cryphonectria* on chestnuts. Isolates collected by Cortesi *et al*. (1997) have been identified as *C. radicalis* based on RFLP and sequences of the ITS region, sequences of the β-*tubulin* gene, and by comparison with isolates reported from Switzerland (Hoegger *et al*., 2002) as being *C. radicalis* (M. J. Wingfield, personal communication). In this study, it was found that *C. radicalis* was common in only one small area in Mt Pelion, close to where it was previously reported by Cortesi *et al*. (1997); it was much less common elsewhere, even in the Mt Pelion region, and only one isolate was sampled in

Macedonia. As in Switzerland, *C. radicalis* coexists on chestnut trees with *C. parasitica*, although at much higher frequencies in two populations. None of these *C. radicalis* isolates was virulent on chestnut seedlings (K. Sotirovski, unpublished data), consistent with previous reports that *C. radicalis* is a weak pathogen (Hoegger *et al*., 2002).

The low diversity of vc types of *C. parasitica* in Macedonia and Greece should make these populations ideal candidates for biological control with transmissible hypovirulence. Based on laboratory studies (Anagnostakis, 1983; Liu & Milgroom, 1996; Cortesi *et al*., 2001), vegetative incompatibility has been thought to be a major constraint on the spread of hypoviruses in the field (Anagnostakis *et al*., 1986; Milgroom, 1995; Milgroom, 1999). To date, *Cryphonectria hypovirus 1* (CHV-1) has been found at high frequencies in some local populations where chestnut trees appear to have fewer cankers and less branch and stem mortality than other stands at comparable times since the initial discovery of blight, although CHV-1 is not widespread in Macedonia (unpublished data). Whether CHV-1 will spread autonomously or can be deployed successfully for biological control remains to be seen. A particular concern, however, is the potential for increase in the diversity of vc types if *C. parasitica* begins to reproduce sexually, e.g. in Glogi where both mating types are present. In that case, 32 vc types could arise by recombination, potentially restricting the transmission of CHV-1 and the prospects for biological control. Future monitoring for changes in vc type diversity and sexual reproduction of this population and others with vc type and mating type polymorphisms is essential for assessing the prospects of biological control with hypovirulence in Macedonia. Measures adequate for preventing the increase in diversity, however, are not currently available.

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